IN THE SPECIFICATION

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As seen in Figures ~~2A~~ 1A & ~~2C~~ 1C, XL-APC has a reduced ration of fluorescence at 650/620 nm versus the native APC. This also is reflected in a reduced ratio of the 650 nm absorbance of APC to the 280 nm absorbance peak reflecting total protein, an indication that the absorbance per molecule has been decreased. One commercially available preparation of XL-APC is called XL665™ (CIS Bio), and this material demonstrates the same decreased ratios of 650/620 fluorescence and 650/280 absorbance observed in the other XL-APC preparations now commercially available.

Page 4, lines 23, 26 and 28

Figure ~~2~~ 1 shows fluorescence emission spectra for native APC (A), SL-APC (B) and XL-APC (C) and its change over time stored at low phosphate concentration.

Figure ~~3~~ 2 shows the change in fluorescence intensity over time at high temperature for native APC, SL-APC and XL-APC.

Figure ~~4~~ 3 shows fluorescence emission spectra for SL-APC, GL-APC and XL-APC stored in dimethyl sulfoxide for varying lengths of time.

Page 5, lines 1, 6 and 9

Figure ~~5~~ 4 shows detection of ~~phoshophorylated~~ phosphorylated poly-GAT in a time-resolved fluorescence energy transfer (TR-FRET) homogeneous assay format with europium labeled anti-~~phoshoptyrosine~~ phosphotyrosine IgG (PY20) as the donor and SL-APC labeled streptavidin as the acceptor compared to a commercially available XL-APC streptavidin as the acceptor.

Figure ~~6~~ 5 Comparison of two tyrosine kinase inhibitors, staurosporine and PP-1, on src tyrosine kinase activity measured in a TR-FRET assay using SL-APC/Europium chelate as the FRET pair.

Figure ~~7~~ 6 Comparison of four different Europium acceptor dyes (XL-APC, SL-APC, PBXL-3 and CryptoFluor-2) for Europium chelate emission in a TR-FRET assay titrating the inhibition of β-insulin receptor tyrosine kinase activity with staurosporine (top graph) and 5-iodotubercidin (bottom graph).

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The SL-APC and GL-APC, when conjugated to streptavidin (SA), gave surprising results in homogeneous time resolved fluorescence (HTRF) experiments on the DISCOVERY microplate reader. These data (as shown in Figure ~~5~~ 4) compare this material to a commercially available XL-APC called XL665. In all cases, the cross-linked SL-APC:SA and GL-APC:SA conjugates of this invention proved to be better than the commercially available XL-APC:SA conjugates in detection of phosphorylated tyrosine standard peptides. In this assay, biotinylated peptides that were phosphorylated at least one tyrosine on each peptide were titrated in a homogeneous assay using either SL-APC:SA of this invention or XL-APC:SA to immobilize the peptide and Eu^{3+} labeled anti-phosphotyrosine IgG to detect the phosphorylated tyrosine on the peptide. On binding of the Eu^{3+} labeled antihosphotyrosine IgG, fluorescent energy transferred to the cross-linked APC from the Eu^{3+} in a time resolved manner.

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A major limitation to APC use in specific binding assays is its poor stability under pigment concentrations useful for these binding assays. Native APC broke down when stored at room temperature at a pigment concentration of 500 ng/mL (Figure ~~2~~1A). This decay occurred more rapidly than the two hour time point presented (data now shown). APC instability was

manifested as a decrease in maximal fluorescence emission intensity and a shift in the fluorescence emission maximum by about 18 nm to the blue.

SL-APC and XL-APC were chemically stabilized to prevent dissociation at the low pigment concentrations normally encountered in a biological assay (Ong & Glazer, 1985). Storage at 500 ng/mL for 2 h at room temperature had little effect on their fluorescence emission spectra in either the absolute peak height or emission maximum (Figures ~~21~~B and ~~21~~C). This increased stability gives the cross-linked APCs the ability to be used in applications for time resolved fluorescence (TRF), flow cytometry and other immunoassay formats.

Chemically cross-linked APC, both GL-APC and SL-APC, has increased thermal stability relative to native APC. This allows the use of GL-APC and SL-APC in applications where the temperature needs to be elevated. Stability studies were carried out at 100 µg pigment/mL in phosphate buffered saline (10 mM phosphate (pH 7.0), 150 mM sodium chloride, 0.05% sodium azide) at 65°C to demonstrate the differences in stability between native APC, SL-APC and GL-APC. SL-APC and GL-APC had comparable temperature stability having little damage for up to 3 days, even retaining 50% of their fluorescent intensity after 60 h incubation at 65°C (Fig. ~~3~~2). In contrast, native APC broke down much more rapidly, losing 50% of its fluorescent intensity by 20 h and about 90% after 45 h.

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In drug screening applications such as time resolved fluorescence, solvents such as DMSO are often employed for sample mobilization. The stability of APC during these screening assays is important for accurate measurement. SL-APC and XL-APC streptavidin conjugates maintain a steady absorbance, providing a constant ability to accept resonance energy transferred from Europium on 10% DMSO treatment. Both dyes maintain their emission maximum at 657

nm. There is an initial decline in fluorescence intensity that quickly stabilizes and then remains relatively constant for several days at this level. Figure ~~4~~ 3 compares the fluorescent stability of GL-APC , a commercial XL-APC and SL-APC over the course of five days. Table 2 compares the change due to 10% DMSO treatment in fluorescence and absorbance over time as a percentage of a control with no DMSO.

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Both GL-APC and SL-APC were tested in 96 and 384 well microplates in time resolved fluorescence energy transfer experiments. The detection of a pre-phosphorylated Poly-GAT peptide was used to compare the performance of XL-APC against SL-APC and GL-APC. In this assay, the APC's were used to capture biotinylated Poly-GAT peptide. A src-tyrosine kinase was used to phosphorylate the peptide, which in turn was detected with Europium labeled Anti-Phosphosotyrosine IgG (PY20) (Park, et al, 1999, *Anal. Biochem.* 269: 94-104). Inhibitors, such as staurosporine, can be added to inhibit phosphorylation to generate classical inhibitor response curves. These data show that a titration in 96 well plates of GL-APC, SL-APC and XL-APC generate comparable data when used in the same system to detect the equivalent amount of peptide (Fig. ~~5~~ 4; Table 3). Even though this assay (in our hands) has not been optimized to minimize non-specific interactions and maximize signal to noise, SL-APC and GL-APC are very competitive with commercial XL-APC conjugates. Table 3B displays signal to noise data generated from a tyrosine kinase assay in 384 well microplates, which provided comparable results to the 96 well format. Further optimization of this assay should provide better signal to noise ratio (s/n) but the relative performance is expected to remain the same.

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Two alternative cross-linked allophycocyanins (XL-APC and SL-APC) were formatted for tyrosine kinase assays in a time-resolved fluorescence energy transfer (TR-FRET) homogeneous assay format using the VictorII® fluorescent microplate reader (PerkinElmer Life Sciences). The dyes were used in a pair of homogeneous kinase assays in 96-well format using the Victor II reader. An initial comparison of the SL-APC streptavidin conjugate in TR-FRET for the src kinase assay demonstrated an improved sensitivity over a commercially available XL-APC streptavidin conjugate (Figure ~~5~~ 4). Since the encouraging initial experimental results from the kinase experiments indicated that these dyes are highly sensitive and stable, a further exploration of the applicability of SL-APC to several tyrosine kinase assays was completed. The SL-APC dye was formatted into tyrosine kinase assays, and it worked very well and could provide advantages over existing dyes on the market.

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Conditions determined for the non-receptor src tyrosine kinase were applied to the insulin receptor tyrosine kinase (ß-IRK). SL-APC was compared to the commercial XL-APC conjugates in the TR-FRET assay (Fig. ~~7~~ 6). The titration of two inhibitors, staurosporine (Hanke, et al., (1996) "Discovery of a novel, potent and Src-family selective tyrosine kinase inhibitor." *J Biol Chem* 271:695) and 5-iodotubercidin (Massillon, et al., 1994 *Biochem* J **299**:123) *versus* the fluorescence intensity demonstrated that all of the dyes worked well in the assay and provided very similar curves. The IC_{50} values derived from the curves were very close for XL-APC and SL-APC, for both inhibitors. The signal intensity and the signal to noise ratios (s/n) with these two tyrosine kinase assay systems utilizing different acceptor dyes is summarized in Table 4. The dyes had signal intensity of the same magnitude, with SL-APC edging out SL-APC in signal intensity.